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Effects of Mutations in Constant Regions 3 and 4 of Envelope of Simian Immunodeficiency Virus

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Twenty-six mutant forms of simian immunodeficiency virus strain mac239 were constructed with changes in constant region 4 (C4) of *env*. Twenty-four of these had a single amino acid change, one had changes in two amino acids, and one had a deletion of eight amino acids. The effects of these mutations on viral replication, gp160 processing, and binding of env protein to soluble CD4 receptor were analyzed. The C4 region was relatively sensitive to sequence changes since only 11 of the 26 mutants replicated appreciably. Eight of the 15 mutants that were replication incompetent exhibited grossly defective processing of the gp160 env precursor; these mutations likely resulted in global effects on gp160 structure. Six of the replication incompetent mutants exhibited normal or near normal gp160 processing and binding of env protein to sCD4 and thus were probably blocked at some step subsequent to binding of virus to its CD4 receptor. Only one of the C4 mutations, 441W → R, resulted in greatly decreased binding to sCD4 while retaining normal processing of gp160. The equivalent residue in HIV-1 has similarly been shown previously to be important for binding of HIV-1 to the CD4 receptor. Since a W → S mutation at position 441 in C4 of SIVmac239 affected both gp160 processing and sCD4 binding, it is not clear whether the 441 tryptophan is actually important for contacting CD4 or for maintaining an appropriate configuration. Mutations within a highly conserved GGDPE sequence in C3 of SIVmac239 specifically affected CD4 binding, which is also similar to previous findings with HIV-1. These results demonstrate similar sequence requirements in SIVmac and HIV-1 env for binding CD4, but they raise doubts as to whether C4 sequences are directly involved in the binding.

INTRODUCTION

The envelope glycoproteins of the human and simian immunodeficiency viruses (HIV and SIV, respectively) mediate the attachment of virus to the surface of $CD4^+$ cells (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; Maddon *et al.*, 1986; McDougal *et al.*, 1986). Attachment occurs through the interaction of the viral-encoded virion surface protein, gp120, and the CD4 molecule itself. gp120 is bound to virions in whole or in part though noncovalent interactions with the viral-encoded transmembrane protein, gp41. gp120 and gp41 are synthesized through proteolytic processing of the gp160 precursor encoded by the *env* gene. Virion gp120 and CD4 interaction triggers conformational changes (Sattentau and Moore, 1991) and a fusion reaction between the viral and target cell membranes.

Early results from Lasky *et al.* (1987) demonstrated that sequences in constant region 4 (C4) of HIV-1 gp120 were important for the CD4 binding reaction. A deletion of 12 amino acids from this region led to a complete loss of binding to CD4 and a particular point mutation, Ala to Asp, resulted in a partial loss of binding activity. Also, a monoclonal antibody directed to these sequences was found to neutralize viral infectivity. Based on these results, the C4 region has frequently been referred to as "the CD4 binding domain." Cordonnier *et al.* (1989) subsequently showed that a highly conserved tryptophan residue at position 427 in C4 was important for binding CD4, consistent with previous observations. Much more detailed analyses performed by Olshevsky *et al.* (1990) showed that discontinuous sites scattered across gp120 were critical for binding CD4. In addition to Trp427, Olshevsky *et al.* (1990) defined Thr257, Asp457, Asp368, and Glu370 as critical residues for CD4 binding. The Asp368 and Glu370 are located within a highly conserved GGDPE sequence in C3.

Two independent studies have recently suggested an interaction between C4 and V3 sequences and a role in post-CD4 binding entry events. A 448V → G point mutation in C4 of SIVmac239, which resulted in a block to viral entry subsequent to CD4 binding, could be compensated by a mutation in V3 (Morrison *et al.*, 1993). These results suggested cooperation between V3 and C4 sequences in the process of virus entry. Wyatt *et al.* (1992) analyzed the reactivity of monoclonal antibodies to HIV-1 under a variety of conditions and similarly concluded an interaction between the C4 and V3 regions of HIV-1.

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In this report, we describe the effects of mutations in C4 and in the GGDPE sequence in C3 in SIVmac239. We conclude that the sequence requirements in gp120 for binding CD4 are similar in SIVmac and HIV-1 but suggest that C4 sequences may not be directly involved in the binding.

MATERIALS AND METHODS

Site specific mutagenesis

Oligonucleotides were synthesized on a Cyclone DNA synthesizer (Milligen-Biosearch, Burlington, MA) and purified on oligonucleotide purification cartridges (Applied Biosystems Inc., Foster City, CA). Site-directed mutagenesis was performed by spliced overlap extension–polymerase chain reaction (SOE–PCR) (Ho *et al.*, 1989), using mutagenic internal primer pairs that spanned the 132-bp C4 region of SIVmac239. The random mutagenesis procedure for construction of a library of C4 mutations has been described in detail elsewhere (Morrison and Desrosiers, 1993). Briefly, two populations of PCR fragments that contained mutations in the region of overlap were generated. These fragments were gel purified, combined in equimolar amounts, and used as the template for a second round of PCR using the nonmutagenic outer primers containing *Sph*I and *Cla*I restriction sites. Six additional specific mutations (385D → E, 385D → N, 387E → Q, 434I → T, 441W → S, and 441W → R) were made by recombinant circle PCR mutagenesis (Jones and Howard, 1991), using unique mutagenic primer pairs and the C4 domain cloned into pUC19 as a template. The deletion of eight amino acids in the C4 domain (DEL434/441) and the replacement of the C4 domain of SIVmac239 with that of HIV-1 (SIV/HIV) were also achieved by a variation of the SOE–PCR method (Ho *et al.*, 1989; Horton *et al.*, 1989).

For the PCR reactions, 10 ng of template DNA and 100 ng of each primer were used. The reactions contained 3.5 m*M* $MgCl_2$, 200 μM dNTPs, and 2.5 U *Taq* DNA polymerase. Amplifications were done with reagents from Perkin–Elmer Cetus (Norwalk, CT) in a Perkin–Elmer DNA thermal cycler for 30 cycles (1 min denaturation at 94°, 2 min annealing at 48°, and 2 min extension at 72°) followed by a final extension for 10 min at 70°. The extension time was increased to 5 min in the recombinant circle PCR procedure.

Cloning and sequencing

DNA from PCR was extracted with chloroform:isoamyl alcohol (24:1), precipitated with 3 *M* sodium acetate and ethanol, treated with Klenow DNA polymerase to ensure blunt ends, and cloned into the *Sma*I site of the plasmid pBS(−) (Stratagene, La Jolla, CA). Recombinant plasmids were detected by colony hybridization using nick translation-labeled DNA from the SIVmac239 envelope gene as a probe, and the mutations were confirmed by dideoxy sequencing. A 1.0-kbp fragment was subcloned into the plasmid p239SpE3′ (Regier and Desrosiers, 1990) using the unique *Spe*I and *Cla*I sites in the envelope gene. In some cases, a 0.3-kbp *Bsp*EI–*Cla*I fragment was subcloned when additional mutations were detected upstream of the C4 region. p239SpE3′, which contains the 3′ half of the SIVmac239 provirus, was first modified by deleting cellular sequences at the 3′ end of the proviral DNA and inserting an SV40 replicon containing the origin of replication and enhancer sequences. This plasmid was used as an envelope expression vector in COS-1 cells (Morrison *et al.*, 1993). Intact mutant proviruses were prepared and used as described (Kestler *et al.*, 1990).

Generation of virus stocks and cell culture

Full-length proviral DNA was used to transfect COS-1 cells using the DEAE-dextran method (Cullen, 1987). At 48 hr post-transfection, cell-free virus was harvested from the medium and virus production was measured by assay of the p27 antigen level with a commercial antigen capture kit (Coulter Co., Hialeah, FL). Viral stocks were used to infect the human T-B hybrid cell line CEM × 174. CEM × 174 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS. Culture medium was changed twice weekly, and an aliquot of clarified medium was used to assay p27 antigen levels in cell-free supernatants.

Processing and CD4 binding

Flasks (25 cm^2) of COS-1 cells at 50% confluence were transfected with 1.5 μg of the modified p239SpE3′ plasmids described above. The COS-1 cells were maintained in Dulbecco's MEM supplemented with 10% FCS, penicillin, and streptomycin. At 48 hr post-transfection, the medium was replaced with MEM lacking FCS, cysteine, and methionine, and incubated for 1 hr at 37°. The starvation medium was replaced with 3 ml of labeling medium containing 10% dialyzed FCS and 100 μCi each of [^{35}S]-methionine and [^{35}S]cysteine. The cells were then incubated for 16 hr at 37°, washed three times with phosphate-buffered saline (PBS), and resuspended in lysis buffer (1% Triton X-100, 0.15 *M* NaCl, 50 *M* Tris–HCl, pH 7.4, 5 m*M* EDTA, 1 m*M* PMSF). The lysates were frozen at −70°, thawed, and the cell debris pelleted and discarded. The clarified supernatants were stored at −70°.

One hundred microliters of each lysate was incubated with either PBS or 250 ng of sCD4 (provided by Dr. Raymond Sweet, Smith-Kline Beecham) in PBS overnight at 4°. The lysates were then incubated overnight with either polyclonal anti-SIV serum or monoclonal anti-CD4 antibody (OKT4 or OKT4A, Ortho Pharmaceuticals, Raritan, NJ) bound to a suspension of protein A–Sepharose. Each sample was washed three times in lysis buffer and once in 50 m*M* NaCl, 50 m*M* Tris–HCl, pH 7.2, resuspended in sample dissociation buffer (125 m*M* Tris–HCl, pH 6.8,

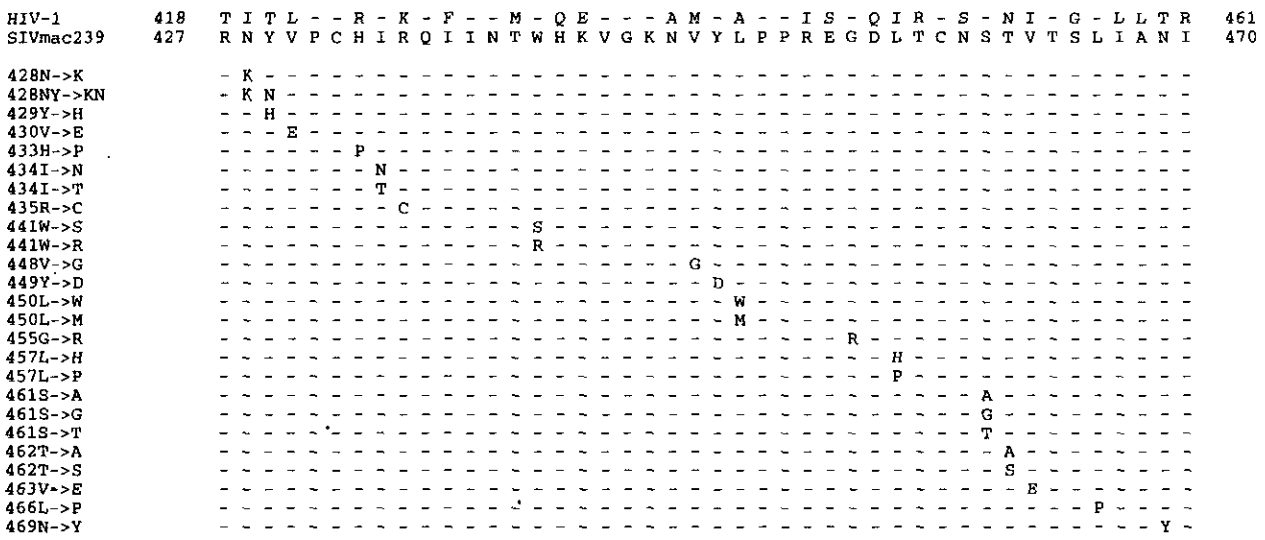

FIG. 1. Amino acid substitutions in the SIVmac239 gp120 C4 domain. SIV numbering is based on Burns and Desrosiers (1991) and HIV-1 numbering is based on Wain-Hobson *et al.* (1985). Dashes indicate amino acid identity.

4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.2% bromophenol blue), and analyzed on 7.5% polyacrylamide–SDS gels.

RESULTS

Mutagenesis

Twenty-four different SIVmac239 mutants with single amino acid changes, one mutant with two amino acid changes, and one with an eight-amino-acid deletion in the C4 domain were selected for study (Figs. 1 and 2; Table 1). In addition to the C4 mutants, three SIVmac239 variants containing changes in the C3 domain (385D → E, 385D → N, and 387E → Q) were generated and analyzed (Fig. 2). We also constructed and analyzed one mutant (SIV/HIV) in which the entire C4 domain of SIVmac was replaced by the corresponding HIV-1 gp120 sequences (Fig. 2). The PCR-derived portion of each mutagenized plasmid was sequenced and the clones used were known to contain only the indicated mutations.

Replication in CEM × 174 cells

Plasmids containing the 3′ half of the SIVmac239 genome with the various modifications in the envelope gene were cut and ligated to the 5′ half of the genome. Virus stocks were generated from transient expression in transfected COS-1 cells. No viral CPE or p27 were detected after infection of CEM × 174 cells with the C3 mutants (385D → E, 385D → N, 387E → Q), the C4 deletion mutant, and the C4 SIV/HIV chimeric virus (Table 1). Of the 25 remaining C4 mutants, 11 (44%) were able to replicate appreciably (Table 1). These 11 mutants contained changes at only 6 different amino acid positions in the C4 domain (Table 1). The remaining 14 noninfectious

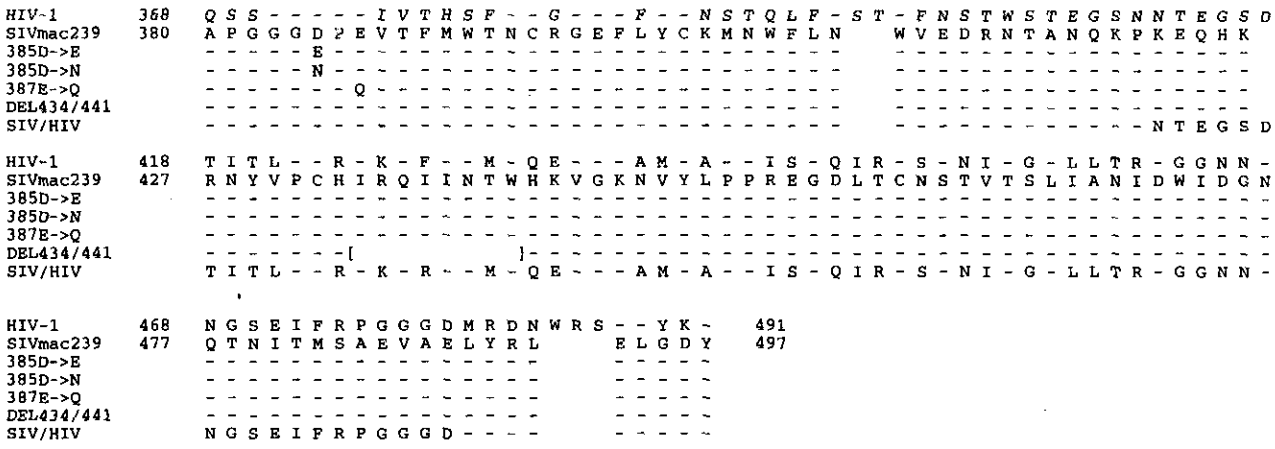

FIG. 2. *Additional SIVmac239 envelope variants constructed by site-directed mutagenesis. As in Fig. 1, an alignment with the corresponding* region in the HIV-1 gp120 is shown at the top. Spaces indicate gaps introduced for optimal alignment. Brackets indicate the position of the eight-amino-acid deletion introduced in the C4 domain.

TABLE 1

REPLICATION AND sCD4 BINDING PROPERTIES OF SIVmac239 ENVELOPE VARIANTS

Variant	Replication in CEMx174[a]	gp160 Processing[b]	sCD4 binding[c]
SIVmac239	+	+	+
428N → K	−	+	+
428NY → KN	−	+	+
429Y → H	−	+	+
430V → E	−	−	−
433H → P	(−)	−	+
434I → N	+	+	+
434I → T	+	+	+
435R → C	−	+	+
441W → S	−	−	−
441W → R	−	+	−
448V → G	−	+	+
449Y → D	−	−	−
450L → W	+	ND	ND
450L → M	+	ND	ND
455G → R	+	ND	ND
457L → H	+	ND	ND
457L → P	−	−	−
461S → A	+	ND	ND
461S → G	+	ND	ND
461S → T	+	ND	ND
462T → A	+	ND	ND
462T → S	+	ND	ND
463V → E	−	−	−
466L → P	−	−	−
469N → Y	−	+	+
385D → E	−	+	−
385D → N	−	+	−
387E → Q	−	+	−
DEL434/441	−	−	−
SIV/HIV	−	−	−

[a] +, Maximal level of p27 was >30% of those obtained after infection with SIVmax239 wild type virus; (−) small amounts of p27 (<1 ng) detected; −, no replication detected.

[b] +, Cleavage of gp160 to gp120 was readily detected when labeled proteins were immunoprecipitated with polyclonal monkey antiserum to SIVmac239. −, Little or no processing of gp160 was detected. ND, not done.

[c] +, gp160 and gp120 were precipitated by OKT4 antibody after binding to sCD4 similar to wild type SIVmac239. −, Binding to sCD4 was not detected. ND, not done.

mutants contained changes at 12 different positions (Table 1). Therefore, this conserved domain in the SIVmac239 envelope seems to be relatively intolerant to change. Some of the amino acid changes (434I → N, 455G → R, 461S → A, 461S → G, 461S → T) which allowed viral replication occurred at positions that are conserved between SIVmac and HIV-1 (Fig. 1 and Table 1).

Some mutants containing changes close to a conserved Cys459 near the carboxy-terminal end of the C4 domain (461S → A, 461S → T, 462T → A, 462T → S) replicated with almost wild-type kinetics (Fig. 3A). However, all of these C4 variants had somewhat lower p27 levels than wild-type at 7 days postinfection and the 461S → G mutant reached only about 30% of the wild-type peak p27 levels. Thus, SIVmac239 containing the original C4 domain seems to have a slight growth advantage compared to these mutants. Some other variants, containing changes closer to the middle of the C4 domain (450L → W, 450L → M, 455G → R, 457L → H), replicated with more delayed kinetics and frequently reached higher p27 peak levels than wild-type SIVmac239 (Fig. 3B and data not shown). This likely resulted from decreased cytopathic effect in CEM × 174 cells and the presence of more viable virus-producing cells in the cultures (data not shown). Another amino acid change close to the amino-terminus of the C4 region (443H → P) reduced viral production about 100-fold (Fig. 3C).

Effects on processing of the gp160 *env* precursor and CD4 binding

We assessed the ability of mutant glycoproteins to bind to the CD4 receptor by measuring the binding of gp160 and gp120 to sCD4. As shown in Fig. 4, both the uncleaved gp160 and the gp120 bound to soluble CD4. The glycoprotein−CD4 complexes could be precipitated by OKT4, which binds to CD4 at a site distinct from the CD4 binding site, but not with OKT4A, which competes for the gp120 binding site (Fig. 4).

Changing two acidic residues in the C3 domain to uncharged residues (385D → N, 387E → Q), or introducing a relatively conservative change of 385D → E retaining the positive charge, resulted in the loss of significant CD4 binding activity (Table 1). These alterations did not interfere appreciably with the processing of the gp160 env precursor (Table 1; Fig. 4A, lanes 5−7). The deletion of amino acids 434 to 441 in the C4 domain, or the replacement of amino acids 422 to 492 of SIVmac239 envelope with the corresponding HIV-1 NL4-3 sequences, resulted in the loss of detectable CD4 binding activity and inefficient proteolytic processing of the mutated glycoproteins (Table 1; Fig. 4C, lanes 7−12).

CD4 binding and gp160 processing were investigated with the remaining 14 replication-incompetent C4 mutants and with 2 of the replication-competent C4 mutants. The gp160 of the two replication-competent mutants (434I → N and 434I → T) was efficiently processed and the mutated glycoproteins bound to sCD4 (Table 1; Fig. 4A, lane 10; Fig 4B, lane 7). Six C4 mutants containing amino acid changes at positions 428N → K, 428/429NY → KN, 429Y → H, 435R → C, 448V → G, and 469N → Y did not replicate detectably in CEM × 174 cells (Table 1), although their glycoproteins bound to sCD4 and were efficiently processed (Table 1; Fig. 4). Six of the single-substitution mutations across the C4 domain at positions 430V → E, 441W → S, 449Y → D, 457L → P, 463V → E, and 466L → P resulted in both inefficient sCD4 binding and inefficient proteolytic processing (Table 1; Fig. 4). One of the SIVmac239 C4 variants, containing a 433H → P change, replicated with very low efficiency in CEM × 174 cells (Fig. 3C). The glycoprotein of this variant was

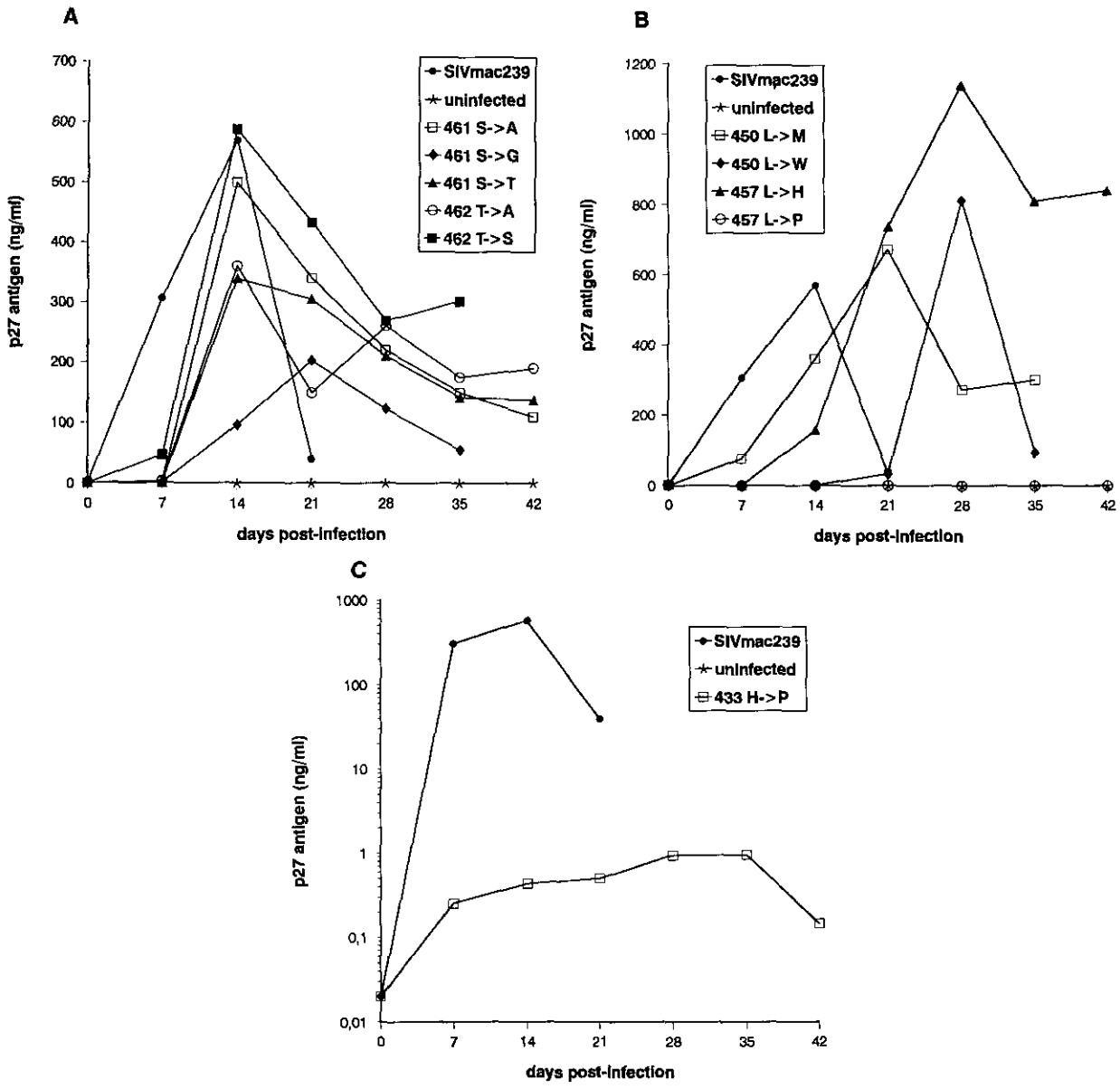

FIG. 3. Replication of SIVmac239 C4 mutants in CEM × 174 cells. Virus stocks were prepared by transient expression in transfected COS-1 cells, and virus containing 0.3 to 2.0 ng p27 antigen was used as an inoculum. Virus production was monitored by assay of p27 antigen at the indicated number of days postinfection. Replication of mutants containing changes at (A) positions Ser461 and Thr462, (B) positions Leu450 and Leu457, and (C) position His433.

only very inefficiently processed and bound to sCD4 with somewhat reduced efficiency (Fig. 4B, lanes 12–14). Finally, the 441W → R mutant was the only one that displayed impaired CD4 binding while retaining efficient proteolytic processing (Figs. 4A and C and Table 1).

Six amino acid positions in C4 (Ile434, Trp441, Leu450, Leu457, Ser461, and Thr462) were changed more than once (Table 1; Fig. 1). Changes in four of these positions did not disrupt viral infectivity. Interestingly, different alterations at the two remaining positions had differential effects. Changing 441W → S impaired sCD4 binding and the proteolytic processing of the gp160 env precursor (Fig. 4A, lane 8, and Table 1). In contrast, the glycoprotein of the 441W → R mutant displayed impaired sCD4 binding but was efficiently processed (Fig. 4A and lane 9; Fig. 4C, lane 2; Table 1). This W residue corresponds to the 427W in HIV-1 gp120 that has previously been shown to be important for binding CD4 (Olshevsky *et al.*, 1990; Cordonnier *et al.*, 1989). This is HIV-1 gp120 residue 432 in the numbering system used in Fig. 1. Changing amino acid 457L → H did not impair viral infectivity (Fig. 4B). However, changing 457L → P resulted in little or no virus replication, inefficient gp160 processing (Fig. 4A, lane 1), and loss of CD4 binding activity (Table 1).

DISCUSSION

Mutation of SIVmac239 385D → E, 385D → N, and 387E → Q in a highly conserved GGDPE sequence in C3

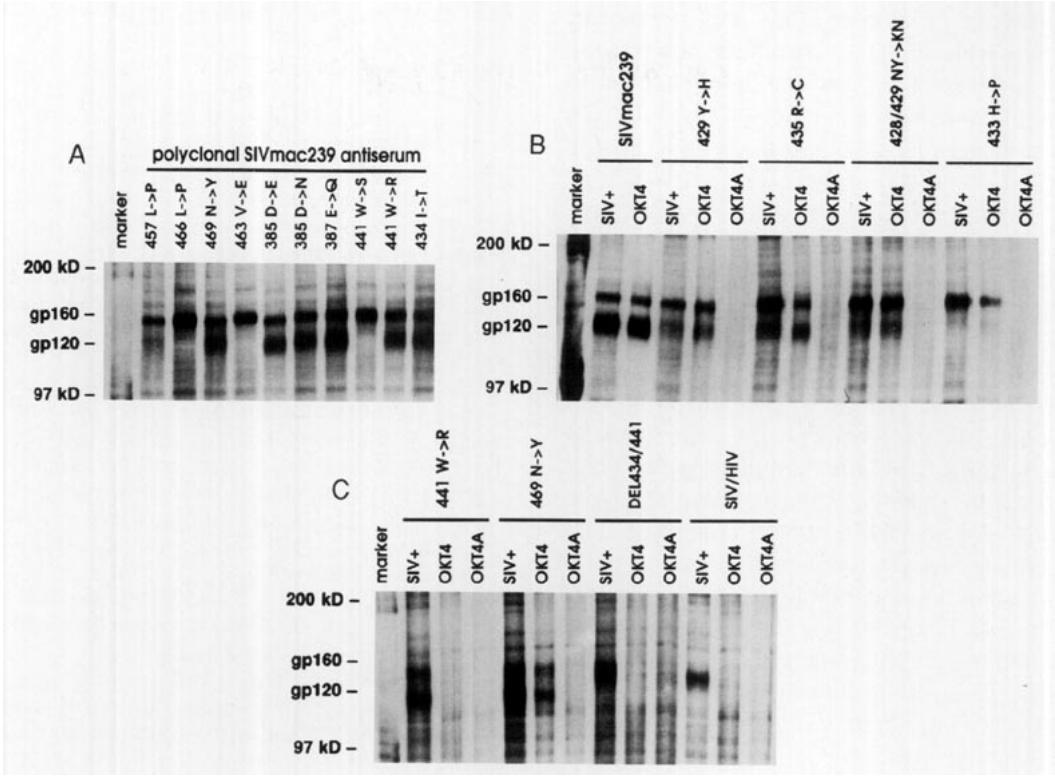

FIG. 4. Proteolytic processing and CD4 binding of mutant envelope proteins expressed in COS-1 cells. Proteins in labeled cell extracts were immunoprecipitated with polyclonal antiserum to SIVmac239 (A, lanes 1 to 10; B, lanes 1, 3, 6, 9, and 12; C, lanes 1, 4, 7, and 10). Labeled cell extracts were also incubated with soluble CD4 and immunoprecipitated with anti-CD4 mAb OKT4 (B, lanes 2, 4, 7, 10, and 13; C, lanes 2, 5, 8, and 11) or anti-CD4 mAb OKT4A (B, lanes 5, 8, 11, 14; C, lanes 3, 6, 9, and 12). (A) Envelope expression and processing in COS-1 cells transfected with mutants 457L → P (1), 466L → P (2), 469N → Y (3), 463V → E (4) 385D → E (5), 385D → N (6), 387E → Q (7), 441W → S (8), 441W → R (9), and 434I → T (10). Envelope expression, processing, and CD4 binding ability of (B) SIVmac239 (lanes 1–2), 429 Y → H (lanes 3–5), 435R → C (lanes 6–8), 428/429NY → KN (lanes 9–11), and 433H → P (lanes 12–14) and (C) 441W → R (lanes 1–3), 469N → Y (lanes 4–6), DEL434/441 (lanes 7–9), and SIV/HIV (lanes 10–12).

specifically abrogated binding to sCD4 without affecting gp160 processing. Mutation of SIVmac239 441W → R in C4 also specifically abrogated binding to sCD4 without affecting gp160 processing. The identical mutations at corresponding locations in HIV-1 have previously been shown to result in the same phenotypic effects (Olshevsky *et al.*, 1990; Cordonnier *et al.*, 1989). These results indicate that SIVmac and HIV-1 have similar sequence requirements for binding to the CD4 receptor. This is consistent with other emerging data that suggest similar sequence–function relationships in SIVmac and HIV-1 envelope proteins. The envelope glycoproteins of these viruses share 30–40% amino acid sequence identity. The location of cysteine residues, which are believed to lock gp120 into a basic configuration (Leonard *et al.*, 1990), are highly conserved between SIVmac and HIV-1 and this in turn is thought to result in a similarity in overall structure (Hoxie, 1991). There is also a striking correspondence of variable and conserved domains (Burns and Desrosiers, 1991) and one consequence of amino acid changes in variable domains is escape from ongoing neutralizing antibody responses (Burns *et al.*, 1993; Choi *et al.*, 1994), a general feature of persisting lentiviral infections (Burns and Desrosiers, 1994). The "V3" domain of SIVmac is now known to be critical for virus entry and for determining tropism (Kirchhoff *et al.*, 1994; Hirsch *et al.*, 1994; Kodama *et al.*, 1994), again similar to that seen in HIV-1 (Freed *et al.*, 1991; Bergeron *et al.*, 1992; O'Brien *et al.*, 1990; Hwang *et al.*, 1991). Finally, independent experimental approaches have indicated an interaction and cooperation between V3 and C4 domains in the process of virus entry for both HIV-1 and SIVmac (Wyatt *et al.*, 1992; Morrison *et al.*, 1993). While subtle differences may exist in how these envelope glycoproteins do what they do, the sequence–function relationships appear for the most part to be fundamentally similar.

A variety of phenotypic effects were found to result from mutations in C4 of SIVmac239. Eight of the 15 C4 mutants that were replication incompetent exhibited grossly defective processing of the gp160 env precursor. Processing of the env glycoprotein precursor is known to be sensitive to changes in structure (Olshevsky *et al.*, 1990; Wiley *et al.*, 1991; Travis *et al.*, 1992) and it seems likely that these eight inactivating mutations affected gp160 processing through changes in the configuration of the molecule. Thus, the structure of the env polyprotein appears to be very sensitive to sequence changes in C4. Six of the 15 replication-incompetent mutants exhibited

approximately normal binding of sCD4 and processing of gp160 and thus were probably blocked at some steps subsequent to binding of virus to its CD4 receptor. In fact, one of the mutants in this category, 448V → G, has previously been shown to be blocked for early stages including entry at some step subsequent to binding of CD4 (Morrison *et al.*, 1993). Only one of the C4 mutations, 441W → R, resulted in greatly decreased binding to sCD4 while retaining normal processing of gp160.

Four aspects of our results lead us to suggest that C4 sequences may not be directly involved in contacting or binding CD4. First, only 1 of the 25 C4 mutants examined had the phenotype of normal gp160 processing and altered CD4 binding. If sequences in C4 were actually involved in contacting CD4, we may have expected a larger fraction of the mutants to have this phenotype. Second, the one residue where such a phenotype was observed, 441W, is probably important for maintaining gp160 structure since the independent 441W → S mutation resulted in loss of gp160 processing. Third, since most of the mutations in C4 appeared to affect structure based on gp160 processing defects, the basis for the defect in the 441W → R mutation must remain suspect. Finally, good evidence has already been presented for a separate, but not necessarily independent, functional role for C4 in cooperating with V3 in post-CD4-binding entry events.

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